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Norbin is an endogenous regulator of metabotropic glutamate receptor 5 signaling

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SUMMARY

Metabotropic glutamate receptor 5 (mGluR5) is highly expressed in the mammalian central nervous system (CNS). It is involved in multiple physiological functions and is a target for treatment of various CNS disorders, including schizophrenia. We report that Norbin, a neuron-specific protein, physically interacts with mGluR5 in vivo, increases the cell surface localization of the receptor and positively regulates mGluR5 signaling. Genetic deletion of Norbin attenuates mGluR5-dependent stable changes in synaptic function measured as long-term depression or long-term potentiation of synaptic transmission in the hippocampus. As with mGluR5 knockout mice or mice treated with mGluR5 selective antagonists, Norbin knockout mice showed a behavioral phenotype associated with a rodent model of schizophrenia, as indexed by alterations both in sensorimotor gating and psychotomimetic-induced locomotor activity.

Keywords

Norbin; neurochondrin; mGluR5; calcium oscillation; NMDA; LTD; LTP; schizophrenia

In view of the numerous important roles of mGluR5 in the CNS (1–[5]), we searched for endogenous regulators of this receptor. We used the carboxy-terminal domain of mGluR5a (Ala^{826}-Leu^{1171}) as bait in a yeast two-hybrid screen. Sixteen interacting clones (26) were isolated, including several known mGluR5-interacting proteins, such as Homer1 (6) and calmodulin (7). Three clones encoded the C-terminus of Norbin (Fig. 1A upper panel and Fig. S1A) (8). Norbin, also known as Neurochondrin (9), is a 75-kD neuronal protein without any known functional domain (10). When tested with all known mGluR receptors (mGluR1 to mGluR8) (11), Norbin (Glu^{499}-Pro^{729}) specifically interacted with a subset of group I mGluRs, namely mGluR1a, mGluR5a, and mGluR5b (Fig. 1A lower panel).

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Direct interaction of Norbin and mGluR5 was confirmed by GST (glutathion S-transgerase) pull-down (Fig. S1B) and co-immunoprecipitation experiments (Fig. S1C). Endogenous Norbin and mGluR5 proteins co-immunoprecipitated from rat brain lysates (Fig. 1B). Experiments with truncated mutants of mGluR5 indicated that the membrane proximal region of mGluR5a (Ala^{826}-Gly^{934}) interacted with Norbin (Fig. S2A). Further studies narrowed the binding sites to two small regions, region A (Arg^{857}-Arg^{867}) and region B (Gly^{893}-His^{903}) (Fig. S2B). Synthetic peptides covering either region A or B interfered with the interaction between mGluR5 and Norbin in cell lysates (Fig. S2C). Replacement of the key amino acids in either region A (mGluR5-mut1) or B (mGluR5-mut2) or both (mGluR5-mut1/2) with alanine abolished binding of mGluR5 to Norbin (Fig. S2D). Norbin binding regions partially overlapped with identified calmodulin binding sites (Fig. S3A and B) (7). However, mGluR5-mut1 is defective in Norbin binding (Fig. S2D), but not in calmodulin binding (Fig. S3C), and therefore was used to study the specific role of Norbin in the regulation of mGluR5 function. Homer and Norbin did not affect each other's binding to mGluR5 (Fig. S3D).

Using an affinity-purified antibody to Norbin (Fig. S4A), prominent expression of Norbin in adult mouse brain was observed in the hippocampus, amygdala, septum, and nucleus accumbens, with moderate expression in the dorsal striatum (Fig. 1C). This distribution of Norbin resembles that of mGluR5 (12). A synaptosomal fraction purified from mouse brain contained both Norbin and mGluR5 (Fig. 1D). In primary hippocampal neurons, Norbin and mGluR5 were localized together in a punctate appearance in dendrites (Fig. 1E). Double staining with antibodies to microtubule-associated protein 2 (MAP2) or spinophilin (Fig. 1F) indicated that Norbin and mGluR5 were localized with the dentritic spine marker spinophilin.

We assessed the physiological consequence of Norbin expression on signaling through the mGluR5 receptor. mGluR5 is coupled to the heterotrimeric guanine nucleotide-binding protein (G protein) α subunit $G_{\alpha q}$ that activates phospholipase C, causes generation of inositol 1,4,5-triphosphate ($InsP_3$), and leads to calcium release and calcium oscillations (1,[13]). After a 30 min exposure to a group I mGluR agonist (10 μM L-Quisqualic acid), more inositol phosphates were formed in HEK293T cells doubly transfected with mGluR5 and Norbin (84.6±5.7% increase above basal level) than in cells transfected with mGluR5 alone (57.0±3.1% increase above basal level) (Fig. S4B). Activation of mGluR5 leads to ERK1/2 phosphorylation and this was also enhanced when Norbin was co-expressed in HEK293T cells (Fig. S4C).

We tested whether Norbin transfection affected mGluR5-elicited calcium oscillations in HEK293 cells stably expressing mGluR5. The average length of the calcium oscillations was significantly longer in Norbin expressing cells than in control cells (12.5±0.8min versus 8.2 ±1.1 min, $p<0.05$, Wilcoxon rank sum test) (Fig. 2A and B). The mean number of calcium peaks was significantly higher in Norbin expressing cells (11.2±0.9 peaks; n=196) than in control cells (6.7±0.8 peaks; n=105; $p<0.05$, Wilcoxon rank sum test) (dashed lines in Fig. 2B). Additionally, 16% of the control cells failed to respond to DHPG, whereas only 7% of the Norbin transfected cells failed to do so (Fig. 2B bottom yellow and blue bars). However, the effect of Norbin was not seen in cells expressing the Norbin-binding defective mutants, mGluR5-mut1 and mGluR5-mut1/2 (Fig.2C).

The fact that Norbin binds to the membrane proximal region of mGluR5 prompted us to test whether Norbin might influence cell surface expression of mGluR5. The amount of cell-surface mGluR5 was significantly increased in the presence of co-transfected Norbin in N2a cells, whereas the amounts of mGluR5-mut1 or mGluR5-mut1/2 (which do not bind to Norbin) at the cell surface were not affected (Fig. 3A). When endogenous Norbin expression was decreased in primary cortical neurons with a specific shRNA (Fig. S5A), the amount of mGluR5 at the cell surface was reduced (Fig. 3B and Fig. S5B).

To further evaluate the role of Norbin in regulation of mGluR5 signaling, we generated Norbin conditional knockout (KO) mice (Fig.S6A and (26)), with deletion of the Norbin gene specifically in the postnatal forebrain (Fig. S6B–D) (14). The abundance of mGluR5 at the cell surface was assayed in wild-type ($Norbin^{Flox/Flox}$) and Norbin KO ($Norbin^{Flox/Flox;\ iCre}$) mice by radioligand binding. Significantly less tritiated MPEP (2-methyl-6-(phenylethynyl)pyridine hydrochloride), a specific mGluR5 antagonist, was bound to membrane fractions from Norbin KO mice than to membranes from wild-type mice (Fig. 3C), whereas total amounts of mGluR5 in the forebrain were not affected (Fig. S6E). The amount of mGluR5 on the surface of cultured primary cortical neurons from Norbin KO embryos was also significantly reduced (Fig. 3D).

mGluR5 is important for synaptic transmission and synaptic plasticity. Depletion of Norbin did not alter basal synaptic transmission or short-term plasticity in hippocampal Schaffer collateral–CA1 synapses (Fig. S7A and S7B). Activation of mGluR5 induces long-term depression (LTD) in the CA1 region (15). Norbin KO mice showed reduced DHPG-induced LTD (85.4±1.4 % of pre-DHPG baseline, n=6), compared to that of wild-type mice (69.9±1.7 % of pre-DHPG baseline, n=8) (Fig. 4A). Activation of Group I mGluRs is necessary for another form of synaptic plasticity, long-term potentiation (LTP), in the CA1 region. Deletion of mGluR1 or mGluR5 reduces LTP (16–[18]). Knockout of Norbin abolished the induction of LTP in the Schaffer collateral–CA1 synapses (Fig. 4B).

Prepulse inhibition of startle (PPI) is a phenomenon in which a reaction (startle) induced by a strong startling stimulus (pulse) is inhibited by a weaker prestimulus (prepulse). Defects in PPI reflect abnormalities of sensorimotor gating, a clinically important feature of schizophrenia (19). mGluR5 positively regulates the function of the NMDA (N-methyl-D-aspartate) receptor in the CNS (20). Consistent with the NMDA hypofunction hypothesis of schizophrenia (21), mGluR5 KO mice display a disruption in PPI (22). We therefore tested PPI in Norbin KO mice. Compared to wild-type littermate controls, Norbin KO mice showed impaired PPI at a pre-pulse intensity of 74 dB (4 dB above background noise) (Fig. 4C upper panel). There was no significant difference in baseline startle response between these two groups (Fig. 4C lower panel).

Similarly to mice treated with the mGluR5 antagonist MPEP (23–[25]), Norbin KO mice showed more locomotor activity in response to MK-801, an NMDA antagonist, than did wild-type controls. There was no difference in baseline locomotor activity observed between wild-type and Norbin KO mice (Fig. 4D and Fig. S8A). MPEP augmented the locomotor-stimulating effect of MK-801 by 109% in WT mice, but had no effect in Norbin KO mice (Fig. 4E and Fig. S8B). These results strongly suggest that the hypersensitity of Norbin KO mice to MK-801 is due to reduced mGluR5 signaling.

In summary, a variety of cell biological, electrophysiological and behavioral studies indicate that Norbin is an important endogenous modulator of mGluR5 and may provide a therapeutic target for schizophrenia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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26. Materials and methods are available as supporting material on *Science* Online.

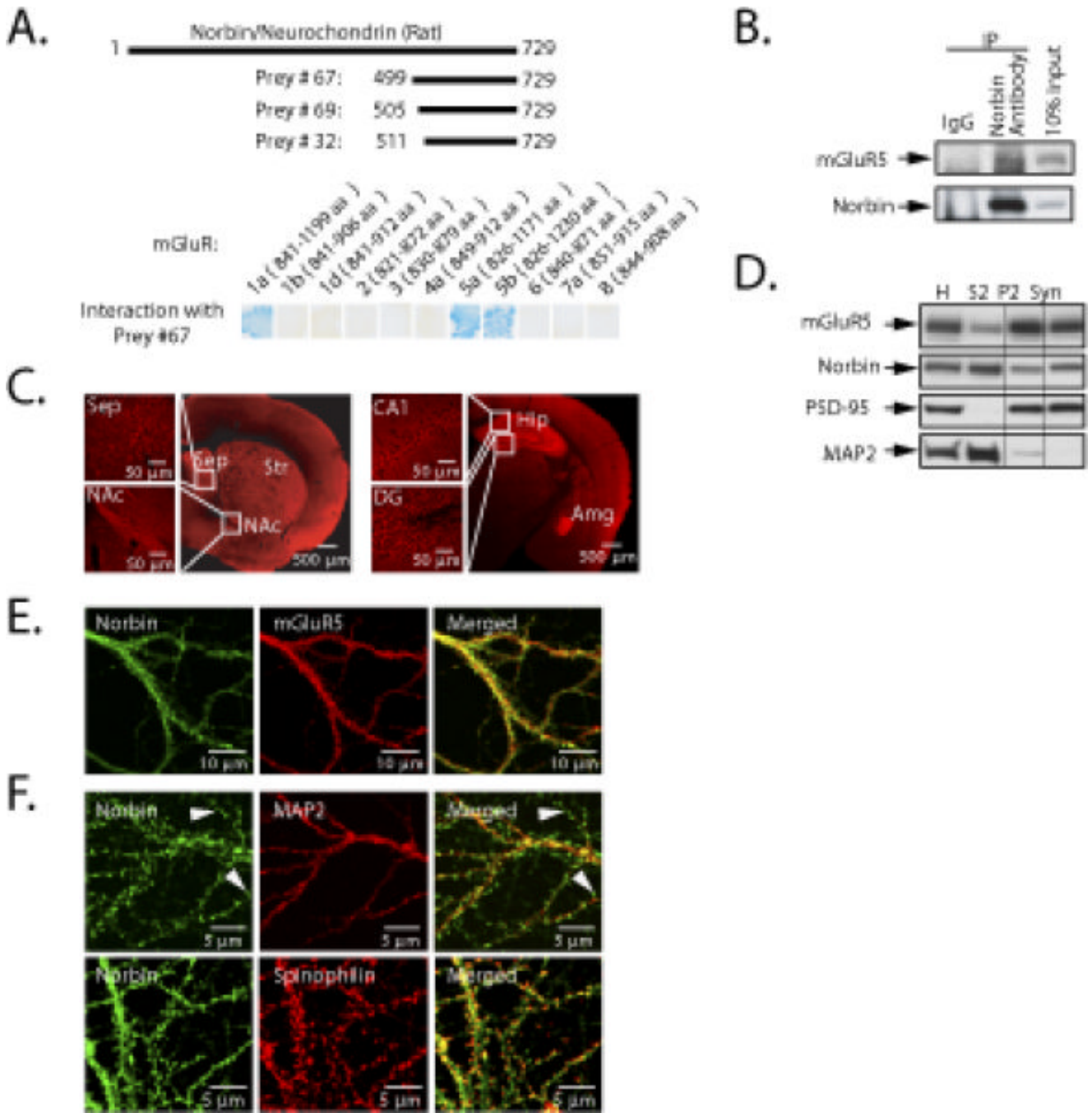

Figure 1. Analysis of the interaction between Norbin and group I mGluRs

(A). **Upper panel**: yeast two-hybrid screen using the cytoplasmic tail of mGluR5a (826–1171 a.a.) as bait identified three partial cDNA clones of Norbin (preys #32, 67, 69). Numbers represent corresponding amino acid residues. **Lower panel:** cytoplasmic tails of mGluR1–8 (as indicated) were co-expressed with Norbin C-terminus (499–729 a.a.) in yeast two-hybrid system and their interactions were tested by β-galactosidase assay. **(B).** Rat hippocampal homogenate was subjected to co-immunoprecipitation using purified antibody to Norbin or control normal rabbit IgG. Samples were analyzed by western blotting using either anti-mGluR5 (upper panel) or anti-Norbin (lower panel) antibodies. **(C).** Coronal slices (50 μm) of adult mouse brain were fluorescence immunostained with antibody to Norbin. Septum (Sep),

nucleus accumbens (NAc), striatum (Str), hippocampus (Hip), dentate gyrus (DG) and amygdala (Amg) are shown with higher magnifications in the insets. **(D).** Synaptosomal fractions (Syn) from mouse brain homogenate (H) were analyzed by western blotting. S2 and P2, supernatant and pellet after 15,000g centrifugation. **(E and F).** Cultured DIV21 hippocampal neurons were fixed and double immunostained with antibodies to Norbin and to mGluR5 (labeled by Zenon Alexa Flour 568) (E); and with antibodies to Norbin, to dendritic marker MAP2, or to the spine marker spinophilin (F).

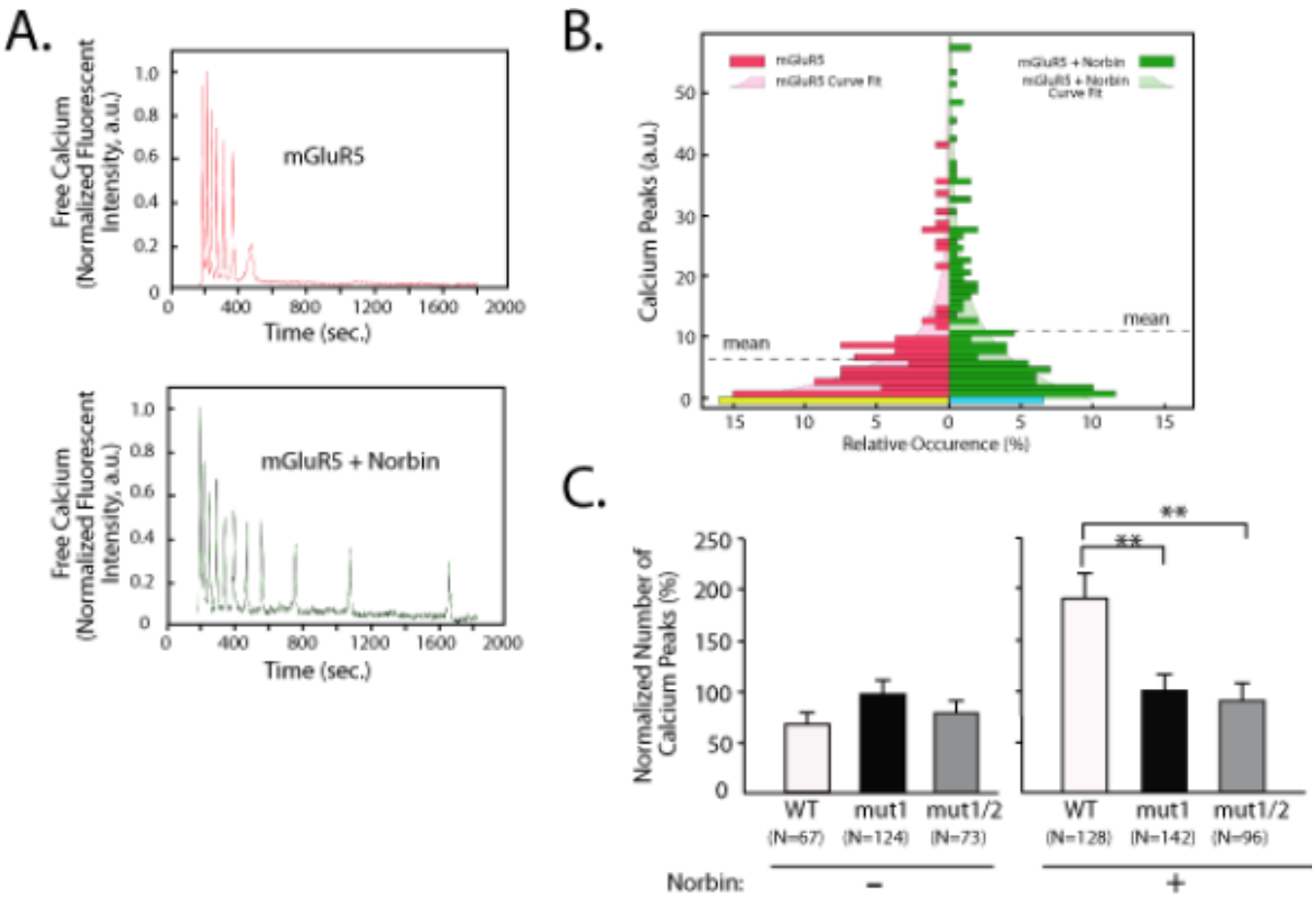

Figure 2. Effects of overexpression of Norbin on mGluR5 signaling

(A). HEK293 cells stably expressing mGluR5 were transiently transfected with Norbin and ratiometric calcium imaging following exposure to DHPG (10 μM) in cells not expressing (upper) or expressing (lower) Norbin is shown. **(B).** The relative distributions of the number of calcium peaks during 28 minutes of continuous exposure to 10 μM DHPG in cells not expressing (pink) or expressing (green) Norbin. The yellow and blue bars at the bottom of the figure show the number of cells that failed to respond to DHPG. The mean numbers of calcium peaks are displayed as dashed lines. **(C).** HEK293 cells were transiently transfected with mGluR5-WT, mGluR5-mut1 or mGluR5 mut1/2 alone (left panel) or together with pEGFP-Norbin (right panel). The number of calcium peaks during 20 minutes of continuous exposure to 10 μM DHPG was recorded. Only cells responding to treatment were included and the data were normalized to the mean number of peaks of cells transfected with mGluR5-mut1. Data represent means ± SEM (**p<0.01, Wilcoxon rank sum test).

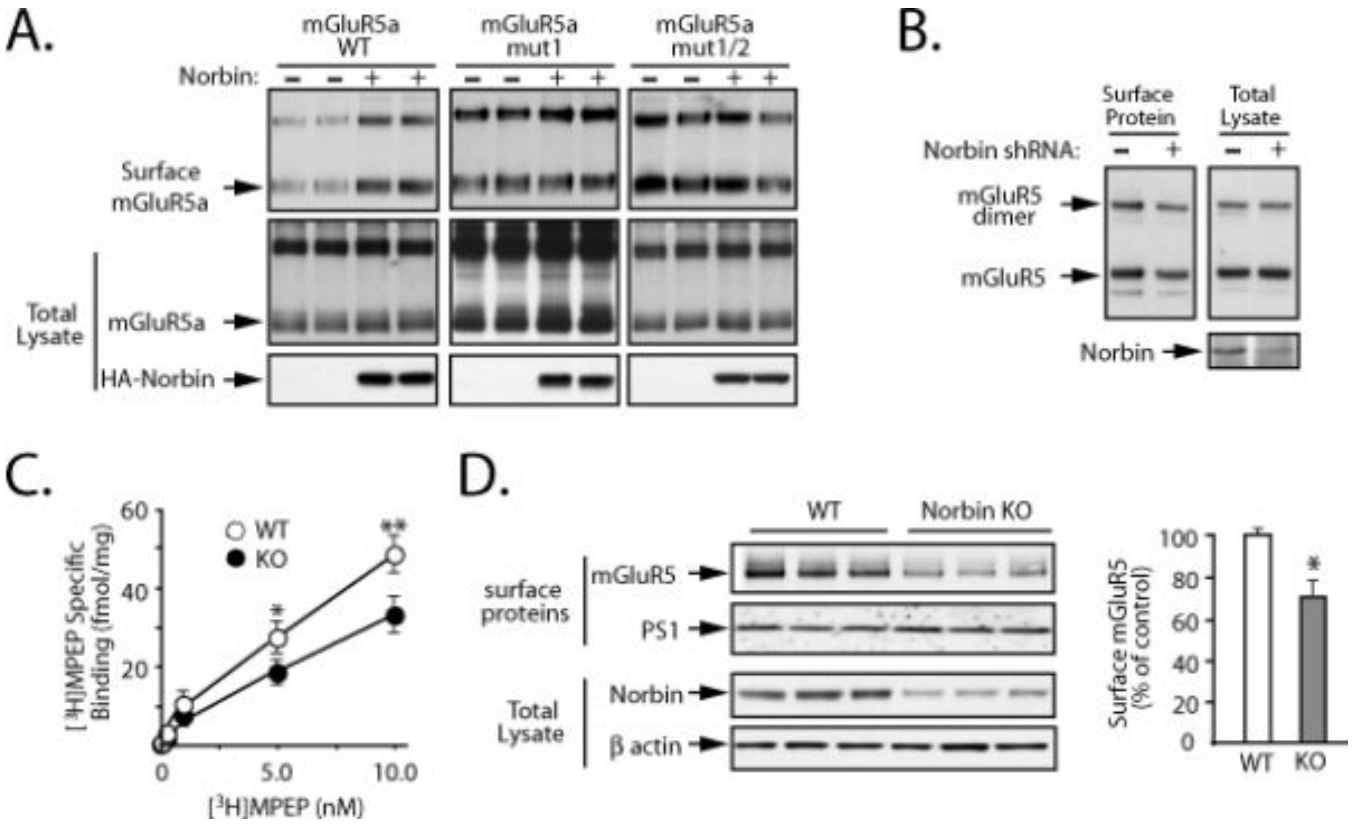

Figure 3. Effects of Norbin on the abundance of mGluR5 at the cell surface

(A). Biotinylated cell surface proteins from N2a cells transfected with myc-mGluR5 (wild-type, mut1 or mut1/2) and vector or HA-Norbin (upper) and total lysates (lower) were analyzed by western blotting using antibodies to myc and HA. **(B).** shRNA-724 targeting Norbin or control shRNA was transfected into E18 cortical neurons using Nucleofector Kit. Five days after transfection, cell surface mGluR5 level was examined by surface biotinylation experiments. **(C).** Binding of [^{3}H]MPEP to membrane fractions from forebrain of WT (○) and Norbin conditional knockout mice (●). Means ± SEM (n=4, *p<0.05 and **p< 0.01, Student's t-test). **(D).** E18 primary cortical neurons isolated from wild-type and Norbin knockout embyos were subjected to surface biotinylation experiments on DIV7. Biotinylated mGluR5 was detected by western blotting. Presenilin (PS1) was used as control surface protein and β-actin was used as loading control. Right panel: quantification of results from four independent cultures. Data represent means ± SEM (*p<0.05, Student's t-test).

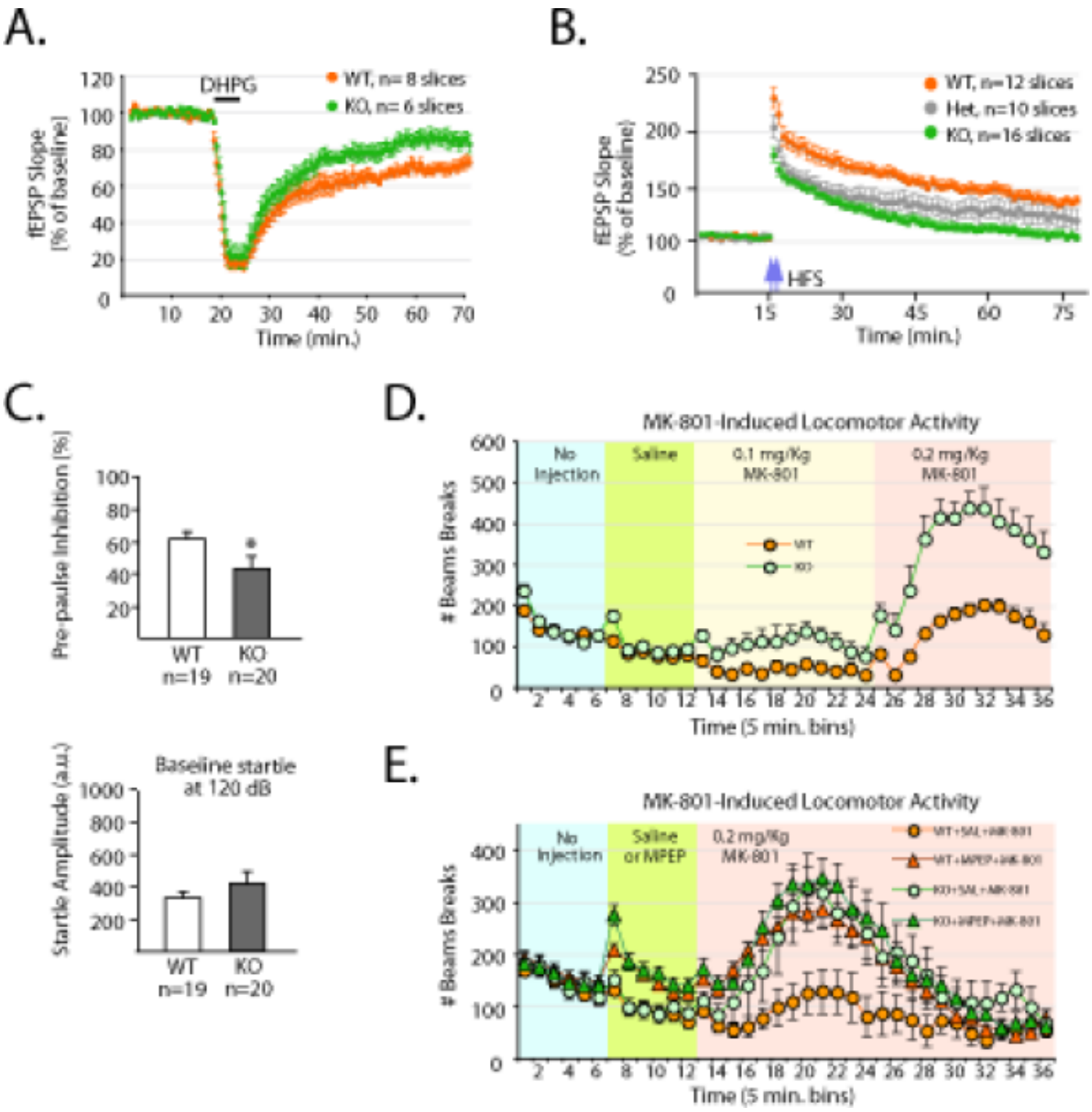

Figure 4. Reduced DHPG-induced LTD, impaired LTP and schizophrenia-like behaviors in Norbin knockout mice

(A). Averaged slopes of fEPSPs were plotted (percentage of pre-DHPG baseline) as a function of time at Schaffer collateral–CA1 synapses before and after DHPG (100 μM, 5 min as indicated by the black bar) treatment for Norbin WT (n=8 slices) or Norbin KO (n=6 slices) mice. Data are means ± SEM. **(B).** LTP analyzed as averaged slopes of fEPSPs at Schaffer collateral–CA1 synapses before and after tetanic stimulation (blue arrows) for WT (n=12 slices), Norbin KO (n=16 slices) or heterozygous (Het) (n=10 slices) mice. Data are means ± SEM. **(C).** Upper panel: pre-pulse inhibition of startle in WT (n=19) and Norbin KO mice (n=20). Mice received prepulses of 74 dB and pulses of 120 dB. Data are means ± SEM.

*p<0.05, Student's *t*-test. Lower panel: average startle magnitudes at 120 dB. **(D).** WT (n=8) and Norbin KO (n=8) mice were successively injected with saline, 0.1 mg/kg and 0.2 mg/kg MK-801 at the indicated times. Locomotor activity was measured by number of beam breaks. **(E).** WT and Norbin KO mice were injected with saline (WT n=10 and KO n=10) or MPEP (WT n=12 and KO n=10) and 0.2 mg/kg MK-801 successively at the indicated times. Locomotor activity was measured by number of beam breaks.